Thermophilic and mesophilic temperature phase anaerobic co-digestion (TPAcD) compared with single-stage co-digestion of sewage sludge and sugar beet pulp lixiviation.

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Abstract

The performance of temperature phase anaerobic co-digestion (TPAcD) for sewage sludge and sugar beet pulp lixiviation (using the exchange process of the digesting substrate between spatially separated thermophilic and mesophilic digesters) was tested and compared to single-stage mesophilic and thermophilic anaerobic co-digestions. The volatile solids removal efficiency from the TPAcD system was dependent on the sludge exchange rate, but was in the range 72.6–64.6%, which was higher than 46.8% with single-stage thermophilic digestion as well as 40.5% with mesophilic digestion. The specific methane yield was 424-468 ml CH_4 per gram volatile solids removed and similar to single-stage mesophilic anaerobic digestion. The increase in microbial activity inside the reactor was directly proportional to the organic loading rate (OLR) (or inversely proportional to the HRT) and inversely proportional to the size of the microbial population in single-stage anaerobic co-digestion systems.

Keywords: Two-phase anaerobic co-digestion, sewage sludge, sugar beet pulp lixiviation, microbial activity

1. Introduction

Single-stage mesophilic completely mixed anaerobic digestion has been widely used for the reduction in volume of organic sludge from wastewater treatment processes and for obtaining energy in the form of methane gas. Mesophilic digestion with sewage sludge usually requires over a 20-day retention time, but it is not so efficient in the reduction of volatile solids and the deactivation of pathogenic organisms. To overcome these limitations, interest in thermophilic digestion and co-digestion has increased in recent years.

Co-digestion is the simultaneous anaerobic digestion of a mixture of two or more substrates. The technology is similar to anaerobic digestion, but it is an attractive option due to the increase in methane yields, because of the positive synergism established in the digestion medium. This fact that increases the economic viability of biogas plants [1].

Co-digestion technology could lead to the following benefits [1, 2]: (1) dilution of inhibitory and/or toxic compounds, (2) increase of the organic content inside the digester, with better utilization of the digester volume, (3) enhancement of digestate stabilization, (4) accomplishment of the required moisture content in the digester feed, with easier handling of blended wastes, (5) a greater reduction in the emission of greenhouse gases to the atmosphere and (6) economic advantages from sharing equipment and costs. However, some drawbacks exist as well: (1) the high cost of waste transfer from the cosubstrate generation point to the anaerobic plant, and (2) the harmonization of different policies regarding waste generators.

To overcome the limitations of mesophilic digestion, interest in thermophilic digestion, using the higher metabolic rate of thermophilic microorganisms, has increased [3-5].

Although better performance in the reduction of volatile solids and the deactivation of pathogenic organisms can be obtained from thermophilic digestion, the effluent quality and ability to dewater the residual sludge are poor, and require additional energy to heat the digester [4, 6]. Especially, thermophilic digestion is not much more sensitive to operational conditions, such as temperature, and the organic loading rate, as well as to the characteristics of the influent sludge [6, 7]. Generally, anaerobic processes can be characterized by the digestion environment, microorganisms and process configuration, and each process has its unique advantages.

According to previous studies [8, 9], two-phase or two-stage anaerobic processes have shown good performance in terms of effluent quality, methane yield, volatile solids reduction and process stability. This implies that the performance of an anaerobic process could be improved with the proper combination of anaerobic process characteristics. Recently, the temperature phased anaerobic digestion (TPAD) process, which consists of thermophilic and mesophilic digesters in series, has been studied in order to incorporate the advantages of both thermophilic and mesophilic digestion [10-12]. The TPAD process can be operated at higher loading rates compared to single-stage processes [11, 13] and is better for the deactivation of pathogenic organisms [13] and in its ability to absorb shock loadings, like other two-stage or two phase anaerobic processes [14]. The first-stage of the TPAD process is sensitive to environmental conditions, and has a notable influence on the overall TPAD process. In addition, the degree of maximum volatile solids reduction and specific methane yield obtainable from the TPAD process are not much different from that of single-stage anaerobic processes with sufficient solid retention time [11]. Recently, phased anaerobic

75 digestion systems have gained attention as a sustainable technology for sludge
76 digestion and methane production [15].
77 Although large-scale TPAcD systems have not been applied widely, researchers have
78 demonstrated the potential superiority of TPAD systems over single-stage digesters
79 and other AD processes. Improved total volatile solids (TVS) and pathogen removal,
80 increased methane yield, process stability and organic loading rate (OLR), a shorter
81 hydraulic retention time (HRT), as well as decreased foaming and short-chain fatty
82 acids in the effluent are some of the positive aspects of anaerobic co-digestion.
83 Although the determination of the number of microorganisms is important in many
84 microbial ecology studies [16], these studies have not assessed the activities
85 associated with the methanogen population. Microbial activity will correlate with
86 number only as long as the environmental conditions remain constant. Any change in
87 substrate and operating conditions in the reactors will alter these parameters.
88 Microbial number and activity represent distinct ecological parameters.
89 The stability of the system depends on the viable bacterial groups, and HRT is a
90 significant factor in selecting the predominant microbial species [17, 18].
91 Understanding the functioning of anaerobic reactors requires quantitative information
92 on microbial numbers, biomass and activities of the bacterial groups involved in the
93 process. The measurement of biomass as volatile solids is a significant limitation in
94 studies on the kinetics of the process, development, operation and monitoring of
95 reactors. Direct count procedures by microscopy methods yield the highest estimates
96 of members of micro-organisms and are occasionally used for an indirect calculation of
97 biomass. Epifluorescence microscopy is widely used for direct counting of bacteria,
98 since it does not require culturing [19]. A characteristic peculiarity of methanogens is

99 their UV-induced blue-green autofluorescence which permits counting by
100 autofluorescence microscopy [20]. However, this method is subjective: it only shows
101 methanogens with a high content of F420 such as hydrogen-utilizing methanogens;
102 acetate-utilizing methanogens belonging to the genus Methanosaeta cannot be
103 counted at all and the genus Methanosarcina is found in clumps made up of many
104 individual cells. Nevertheless, it is a frequently used method to count autofluorescent
105 methanogens in anaerobic reactors [18, 21].
106 The aim of this research was to test the configuration of anaerobic co-digestion, using
107 a temperature phased anaerobic co-digestion (TPAcD) process which consists of a
108 thermophilic digester followed by a mesophilic one, to improve the efficiency of single
109 phase anaerobic co-digestion of sewage sludge and sugar beet pulp lixiviation. Thus,
110 the performance of the single-stage completely mixed thermophilic and mesophilic
111 digestions were examined and their characteristics compared with the results obtained
112 in temperature phase anaerobic co-digestion. Mesophilic and thermophilic single-stage
113 anaerobic co-digestion for sewage sludge and sugar beet pulp lixiviation were
114 compared between them.
115 Relationships between OLR, methane generation and both methanogenic anaerobic
116 micro-organisms and the activity of those microorganisms were also considered.
117 The most important novelty of the data presented in this study is the direct
118 experimental evidence regarding the influence of HRT on the population levels of
119 methanogenic anaerobic micro-organisms in the digester.
120 **Notations**
121 **AD** Anaerobic digestion
122 **AcD** Anaerobic co-digestion

COD Soluble carbon oxygen demand

CSTR Continuous stirred-tank reactor

HRT Hydraulic retention time

ORL Organic loading rate

SBPL Sugar beet pulp lixiviation

SS Sewage sludge

TPAcD Two phase anaerobic co-digestion

TPAD Two phase anaerobic digestion

TS Total solids

TVS Total volatile solids

VFA Volatile fatty acids

WWTP Waste water treatment plant

2. Materials and methods

2.1. Experimental process

The schematic diagrams of the anaerobic co-digestion systems used for the experiments are shown in Figure 1. For the temperature co-phase anaerobic co-digestion system, (a) the lab-scale system consisted of a 6 l thermophilic reactor (5 l working volume) followed by a 6 l mesophilic digester (5 l working volume). Both experimental digesters shared similar characteristics: the cover of each reactor incorporated three separate ports for different functions: feeding, mechanical agitation, and measurement of biogas generation (using a 10 l Tedlar bag). The reactors were kept at the selected temperature by water circulating in the water jacket surrounding the reactors.

For the single-stage anaerobic co-digestion systems, semi-continuous laboratory-scale stirred tank reactors were used (Figure 1b). The equipment consisted of a reactor with a stainless steel vessel that was agitated and heated. The total volume was 6 l and the working volume was 5 l. ~~To maintain the operating temperature, the digesters were heated by recirculating water through a thermostatic jacket.~~ Biogas was collected in 10-l Tedlar bags, and a special syringe was used for sampling the gases.

Six tests were developed (Table 1).

In TPAcD systems, the thermophilic digester was fed with a mix of sewage sludge and sugar beet pulp lixiviation (50-50 w/w) and the mesophilic digester was fed with the effluent generated in the previous thermophilic digester.

Two TPAcD experiments were carried out. The first-stage thermophilic (55°C) digester was operated at 10 and 6 days of retention time, respectively; its effluent was used to provide feed for the second-stage mesophilic (35°C) digester. The second-stage digester was operated at an HRT of 10 days in both cases. Therefore, two HRT combinations were assayed: 20 (TPAcD10/10) and 16 (TPAcD6/10). Each condition was maintained for an operational period lasting three times the duration of the HRT to ensure that steady state conditions were reached by checking whether constant effluent characteristic values (carbon oxygen demand soluble (COD), total solids (TS), total volatile solids (TVS), gas production and composition, volatile fatty acids (VFA) and alkalinity levels. Sampling during each steady-state period was performed for five consecutive days.

2.2. Anaerobic inocula and substrates

168 The digester was initially loaded with a mixture of inoculum and substrate, resulting in
169 a final concentration of 20% w/w of inoculum, which is considered optimum for biogas
170 production [22].
171 Primary sludge from the WWTP of San Fernando-Cádiz was used as the inoculum in the
172 mesophilic reactor. The mixed anaerobic culture used as the thermophilic inoculum of
173 the CSTR reactor was obtained from a lab digester running at 20 days of HRT. The
174 inoculum was obtained through a direct change from mesophilic (35°C) to
175 thermophilic conditions (55°C), as described by Riau et al. (2010). The characteristics of
176 the inoculum used in the start-up process are presented in Table 2. The substrate was
177 composed of sewage sludge and sugar beet pulp lixiviation.
178 **Sewage sludge:** The digesters were fed with sewage sludge collected from the
179 aforementioned WWTP.
180 **Lixiviation of sugar beet pulp**: Pellets were collected from Azucarera Ebro Company in
181 Jerez de la Frontera (Cádiz). Sugar beet pulp used as the co-substrate was subjected to
182 physical pretreatment before the co-digestion process in order to promote hydrolysis
183 and solubilization of the organic matter and, therefore, improve anaerobic digestion in
184 the generation of biogas and enhance the final residue's agronomic valorization [22].
185 Once the inoculum was mixed with the substrate, i.e. a mixture of sewage sludge and
186 lixiviation of sugar beet pulp, the system remained unfed for a period of one week to
187 acclimatize the inoculum to the waste at the selected temperatures (35 and 55°C).
188 The average feeding compositions for each reactor in all experiments carried out are
189 summarized in Table 3.

Initially, the organic loading rate (OLR) applied to the single-stage thermophilic and mesophilic reactors were 1.2 and 2.1 g TVS/l/d for T20-M20 and T10-M10, respectively.

For TPAcD, the initial OLR applied were 2.2 and 2.5 g TVS/l/d for TPAcD10/10 and TPAcD6/10, respectively.

These conditions were maintained until steady state conditions were reached.

2.4. Chemical and microbial analyses

The volume and composition of biogas were determined daily. The biogas produced was quantified using a gas flow meter (Ritter TG1) and a gas suction pump (KNF Laboport). Gas chromatography was used to analyze the different components of the biogas. The gases analyzed were: H_2, CH_4, CO_2, O_2 and N_2 (GC-2010 Shimadzu).

The following analytical determinations were performed to monitor and control the process in the substrate and the effluent: TS, TVS, pH, soluble COD, alkalinity and VFA (acetic, propionic, iso-butyric, butyric, iso-valeric, valeric, iso-caproic, caproic and heptanoic). The pH was measured daily using a Crison 20 Basic pH meter. TVS, COD and VFA were analyzed three times a week. These determinations were performed according to APHA (1995) [23]. Organic matter removal was calculated as the percentage difference between the TVS of the influent and the TVS of the effluent within the substrate TVS. Total acidity was calculated by addition of the individual fatty acids.

Quantification assays were performed when reactors reached steady-state conditions. The attainment of the steady state was verified after an initial period (three times the HRT) by checking whether the effluent characteristic values continued at the mean of the previous measurements. The autofluorescent methanogens in the reactors were

214 counted by autofluorescence microscopy. The experimental protocol was performed
215 according to Solera et al., 2001 [18].

216 **3. Results and discussion**

217 The operational conditions were applied to reactors in the mesophilic range (M20 and
218 M10), in the thermophilic range (T20 and T10), and temperature phased (TPAcD10/10
219 and TPAcD6/10). The operational conditions are presented in the Table 4
220 Table 5 shows Effluent quality and performance of the single-stage mesophilic and
221 thermophilic co-digestion processes. The data shown the results of the stability period
222 for each HRT studied.

223 **3.1. Single-stage mesophilic and thermophilic digestion**

224 During the operation time of the single-stage anaerobic processes, the alkalinity level
225 of the thermophilic digestion process was higher than that of the mesophilic process,
226 as shown in Figure 2b. It is well-known that alkalinity in an anaerobic digestion can be
227 generated from the degradation of nitrogenous organic compounds, sulfate reduction,
228 the release of orthophosphate and an increase in VFAs [24, 25]. In this study, the
229 ammonia nitrogen from the thermophilic digestion process was 808 mg/l, which was
230 higher than the 707 mg/l in the mesophilic process at 20 days of HRT. The same
231 behavior was observed at 10 days of HRT (Table 5).
232 The pH value of the effluent substrates gradually decreased between 20 days HRT and
233 10 in both temperature regimes, as shown in Figure 2b. Although, the pH values of the
234 mesophilic process at 20 and 10 days HRT were below 7.5, the digestion showed good
235 behavior and was stable at this value. The pH of the thermophilic process was
236 generally higher than that of the mesophilic process. This was a result of the higher
237 alkalinity of the thermophilic anaerobic digestion process. The increased alkalinity and

238 thus pH from the degradation of nitrogenous compounds in our experiments is in
239 agreement with previous studies [26].
240 The COD level of the thermophilic process was much higher than that in the mesophilic
241 process, as shown in Table 5. At the steady state, the mean values of soluble COD were
242 4.9 and 1.6 kg/m^3 for the thermophilic and mesophilic processes, respectively (Table 5)
243 for the optimum hydraulic retention time (10 days). The VFA level in the thermophilic
244 process was generally higher than that in the mesophilic process, which was consistent
245 with the COD data (Figure 2a). This clearly shows that mesophilic digestion was
246 superior to thermophilic digestion in terms of the effluent quality, which can be
247 explained by the low substrate affinity of some thermophilic organisms [4, 6, 7].
248 The main component of VFA in the mesophilic and thermophilic processes was
249 acetate, but in the thermophilic process at 20-days of HRT, propionate was present at a
250 very high value (Figure 2e). Based on the literature [6, 7], the higher level of
251 propionate in the thermophilic digester occurred due to the higher hydrogen partial
252 pressure, and the acetate was from the higher organic loading rate conditions. In this
253 study, the accumulation of propionate in the thermophilic digester was probably due
254 to the wide fluctuation in the influent characteristics. This indicates that acetogens and
255 hydrogenotrophs under thermophilic conditions are more sensitive to environmental
256 changes. At 10 days HRT, the thermophilic process was able to compensate for the
257 variations in feeding because it was working with an optimum organic loading rate.
258 The VFA to alkalinity ratio for the four single-stage anaerobic systems were monitored
259 to compare the buffering capacities for the change in pH (Figure 2.a). It has been
260 reported that the buffering capacity is sufficient when the VFA-to-alkalinity ratio is
261 maintained below 0.4 [10]. In this study, this ratio in the mesophilic process was below

0.1. For the thermophilic anaerobic digestion process, this ratio was a little higher in both HRTs studied in this work. The slightly higher VFA-to-alkalinity ratio of the thermophilic process was primarily a result of the higher VFA concentration. This indicates that single-stage mesophilic anaerobic co-digestion had better buffering capabilities than thermophilic co-digestion.

The performance of the digesters with respect to solids removal for different tests is presented in Table 5 and Figure 2d. For single-stage reactors, thermophilic conditions resulted in higher removal than the corresponding mesophilic operated reactors. There was a noticeable increased in terms of volatile solids removal when the reactor temperature was raised, with removal rates increasing from 40.5 to 76.5% for 10 days of HRT. For a longer retention time (20 days HRT), the difference between the mesophilic and thermophilic regimes was lower since at this HRT the bacteria in the mesophilic range are capable of biodegrading all biodegradable solids, although 20 days is not the optimum retention time. Maibaum and Kuehn (1999) [4] reported that the difference in the degradation rates of solids substrates under thermophilic and mesophilic conditions becomes significant in relation to the decrease in the retention time.

As shown in Table 5, the average methane content of the biogas from the mesophilic process was higher, at around 70%, than that of the thermophilic process. This was probably a result of the reduced solubility of carbon dioxide under thermophilic conditions [26]. In previous studies, the methane content of the biogas was mainly affected by the type of substrate, rather than the temperature conditions, for anaerobic digestion [5, 26]. However, the specific methane yield of the mesophilic process, based on the removed VS, was a little more sensitive to the influent

286 characteristics of feeding, indicating a higher capacity of mesophilic methanogens for
287 coping with variations in influent characteristics compared to thermophilic
288 methanogens. The average specific methane yield of the thermophilic process was
289 lower, at 210 ml CH_4/$gTVS_{removal}$, than the 630 ml CH_4/$gTVS_{removal}$ by the mesophilic
290 digester for the optimum retention time (Table 5). This was presumably due to the
291 higher maintenance energy of the anaerobic thermophilic microorganisms [6, 7], as
292 well as the higher hydrogen content of the biogas [26]. In comparison with the
293 thermophilic reactor, the mesophilic reactor produced a greater quantity of methane
294 per gram of TVS destroyed at the optimum HRT. This suggests that the thermophilic
295 reactor was not efficient in converting all the intermediate products to methane.
296 The biomethanation process involves stepwise degradations of complex biomass by
297 diverse microbial populations that interact with each other. Four guilds of microbes,
298 which include hydrolytic acidogens, non-hydrolytic acidogens, syntrophic acetogens,
299 and methanogens, drive the biomethanation process in a sequential and concerted
300 manner.
301 After the analysis of the single-stage anaerobic co-digestion of sewage sludge and
302 sugar beet pulp lixiviation, the condition employing 10 days as the hydraulic retention
303 time in the mesophilic regime were determined to be the best option. Once this HRT
304 was chosen, the next goal was to compare this optimum with two-phase anaerobic
305 digestion technology.
306 **3.2. The thermophilic and mesophilic co-phase anaerobic digestion**

307 An increase in biogas production was observed in the TPAcD10/10 process: biogas
308 generation increased from 1.70 l/d under thermophilic conditions for 10 days of HRT to
309 3.42 l/d under mesophilic conditions to 3.59 l/d in the temperature phased system.
310 The alkalinity levels of the temperature co-phase thermophilic and mesophilic
311 digesters were influenced by the variation in the alkalinity of the influent substrate, as
312 shown in Figure 3a. The average level of alkalinity in the co-phase thermophilic
313 digester was around 3400–5300 mg/l as $CaCO_3$, which was higher than 3200–3800
314 mg/l as $CaCO_3$ in the co-phase mesophilic digester. The greater alkalinity under
315 thermophilic conditions was similar to that of the single-stage anaerobic processes, as
316 shown in Figure 2b, and reflects the higher degradation activity toward nitrogenous
317 organic compounds, such as proteins, under thermophilic conditions [26]. The pH
318 levels of the co-phase thermophilic and mesophilic digesters in TPAcD10/10 were in
319 the range of the methanogenic process; nevertheless, the pH in TPAcD6/10 decreased
320 to below 7 in the thermophilic range due to VFA accumulation. In the first TPAcD test,
321 the pH levels in the mesophilic and thermophilic digesters were similar to those in the
322 single-stage mesophilic and thermophilic anaerobic processes.
323 The influence of the substrate exchange rate on the pH of the TPAcD system was not
324 observed in the first assay. However, when the HRT in the thermophilic phase was
325 decreased, the accumulation of VFA occurred, causing a decrease in pH in the
326 thermophilic digester and, in addition, a fault in the mesophilic reactor. Therefore,
327 with the substrates used in those test the optimum TPAcD system is TPAcD 10/10
328 where thermophilic reactor is a pretreatment of the mesophilic anaerobic co-
329 digestion.

330 In TPAcD10/10, the VFA values in the co-phase thermophilic digester became stable
331 after the operation time, as well as that of the mesophilic digester, and were not
332 influenced by the wide change in the influent characteristics. At the steady state, the
333 VFA value in the co-phase mesophilic digester was 537 mg AcH/l, which was lower
334 than 761 mg AcH/l found in the mesophilic digester. This indicates that the mesophilic
335 digester of the co-phase system was stable and functioned well. The affinity of the
336 thermophilic substrate for VFA was quite a bit lower than that of the feeding from the
337 single-stage mesophilic digester (Table 5). This seems to suggest that the higher
338 substrate affinity methanogenic bacteria were selected and dominated in the co-phase
339 mesophilic digester by the substrate exchange between the thermophilic and
340 mesophilic digesters. In the case of the co-phase thermophilic digester, the VFA value
341 was slightly higher than that of the single-stage thermophilic digester.
342 In the TPAcD6/10 test, an accumulation of VFA in the thermophilic digester occurred
343 because of the reduced HRT. Due to this circumstance, anaerobic co-digestion in the
344 mesophilic digester failed.
345 The main VFA component of the co-phase mesophilic digester was acetate, as in the
346 single-stage mesophilic process (Figure 2 and Figure 3). However, in the co-phase
347 thermophilic digester, the propionate content was considerable at both HRTs. This
348 higher propionate content (Table 6) at a higher substrate exchange rate in the co-
349 phase thermophilic digester was probably related to the higher hydrogen partial
350 pressure [6, 26].
351 Individual and total VFA concentrations in the effluent of the first-stage reactor
352 increased when the total HRT decreased in each assay. This indicates that the HRT of
353 the thermophilic phase is a more important factor affecting the VFA content.

354 The reduction of HRT in thermophilic reactor of the TPAcD process and the subsequent
355 VFA accumulation conditioned the pH of the digester (Table 6).
356 Table 6 shows the SCOD values of the thermophilic and mesophilic temperature co-
357 phase co-digestion systems. At steady state, in TPAcD10/10, the SCOD values in the co-
358 phase thermophilic and thermophilic digesters were 5800 and 3000 mg/l, which were
359 higher than those of single-stage mesophilic and thermophilic processes, respectively.
360 The good effluent quality in terms of COD was mainly attributable to the low VFA
361 levels in the co-phase thermophilic and mesophilic digesters, probably due to the
362 higher methanogenic activity and higher affinity of the anaerobic substrate for VFA in
363 the co-phase system in the first TPAcD test.
364 Figure 3a shows the VFA-to-alkalinity ratio required to evaluate the buffering capacity
365 of the temperature co-phase anaerobic co-digestion system; values higher than 0.5
366 clearly indicate that the reactor does not contain a good equilibrium between
367 acidogenic and methanogenic microbiota. In TPAcD10/10, the VFA-to-alkalinity ratios
368 were 0.21 for the thermophilic digester and 0.20 for the mesophilic digester, which is
369 an indicator of a high level of stability. These values indicate that the buffering capacity
370 in the temperature co-phase anaerobic system was sufficient for SS and SBPL co-
371 digestion, as with the single-stage mesophilic anaerobic processes. The slightly higher
372 buffering capacity in the co-phase thermophilic digester was attributable to both a
373 higher alkalinity level from the enhanced degradation of nitrogenous compounds and
374 as well as the VFA level. The higher buffering capacity in the co-phase thermophilic
375 digester also contributed to the good buffering capacity in the mesophilic digester
376 through substrate exchange between the thermophilic and mesophilic digesters.

377 Nevertheless, a reduction in the HRT in the thermophilic phase (TPAcD6/10) caused an
378 increase in the acidity/alkalinity ratio in the thermophilic effluent with a value of 0.77.
379 The overall specific methane yields were as good as the single-stage mesophilic
380 anaerobic process (T10), although some portion of the overall yield was from the
381 thermophilic digester of the co-phase digestion system. The HRT of the mesophilic
382 digester was 10 days, in both cases, but the specific methane yield was lower in
383 TPAcD10/10, showing 340 ml CH_4/g$TVS_{removal}$. The methane generated from the wastes
384 calculated with respect to TVS removal was higher in mesophilic phase of the TPAcD
385 process in comparison with the thermophilic stage. This suggests that the thermophilic
386 reactor was not efficient at converting all the intermediate products into methane. In
387 TPAcD6/10, there was a drop in biogas production in the mesophilic phase, due to the
388 accumulation of VFA in the previous stage.
389 The TVS in the co-phase mesophilic and thermophilic digesters were stable, and were
390 not influenced by the TVS variation in the influent substrate, as shown in Figure 3b.
391 The reduction in volatile solids was around 77.2% in TPAcD10/10, and remained stable
392 in TPAcD6/10 although the global methane yield was lower, as shown in Table 6. In the
393 literature [13], the reduction in volatile solids obtained using the TPAcD process for
394 waste-activated sludge was about 50% at 28 days of SRT, which was around 10%
395 higher than that of the single-stage mesophilic digester. In this study, the reduction in
396 volatile solids that could be obtained in the co-phase digestion system was over 36.5%
397 higher than that of the single-stage mesophilic digester and around 1% higher than the
398 single-stage thermophilic digester. The enhanced performance in terms of TVS
399 reduction obtained from the temperature co-phase anaerobic digestion system was
400 mainly attributable to the higher hydrolytic activity of the thermophilic digester. On

401 the other hand, the additional energy for substrate exchange and for heating the
402 thermophilic digester in the co-phase digestion system should be considered.
403 However, these additional energy requirements could be compensated for by the
404 advantages of the co-phase digestion system, including the reduction of volatile solids,
405 better effluent quality and process stability, and increased methane production,
406 compared to the single-stage mesophilic or the thermophilic processes.

407 **3.3. Microbial population dynamics**

408 Microbial populations in anaerobic digestion have previously been investigated, with
409 the finding that HRT is a significant factor in selecting the predominant microbial
410 species [18, 32]. One of the objectives of the present study was to obtain direct
411 experimental evidence for the influence of HRT on the population levels of
412 methanogenic anaerobic microorganisms in the digester.
413 The results show the evolution of the methanogenic bacteria concentration at
414 different HRT (days). The methanogenic counts were performed at the end of each
415 period [19, 20, 33] when the microbial population had adapted to the new organic
416 loading rate conditions in the mesophilic and thermophilic single-stage anaerobic co-
417 digestion process as well as the TPAcD processes (TPAcD10/10 and TPAcD6/10).
418 Anaerobic effluent from the mesophilic anaerobic digester of sewage sludge from a
419 waste water treatment industrial plant was used as the inoculum. In single phase
420 anaerobic co-digestion, the microbial community is not dependent on the imposed
421 OLR. However, the microbial community was larger in the mesophilic range than in the
422 thermophilic range in both HRTs assayed. In the TPAcD process, a slight increase in the
423 microbial population took place, compared with 10 days HRT in mesophilic and

424 thermophilic single anaerobic co-digestion, as the result of the higher content of
425 microorganisms in the substrate.
426 Methanogenic microorganism activity was determined by comparing the amount of
427 methane generated for each HRT tested with the size of the population in the
428 methanogenic reactor analyzed by epifluorescence microscopy. The results are shown
429 in Table 7.
430 Microbial activity increased between 20 and 10 days of HRT in mesophilic and
431 thermophilic single anaerobic co-digestion, and was much higher when the microbial
432 content in the reactor decreased. In systems with no biomass retention, a decreased
433 HRT is reflected by a lower number of microorganisms exiting the system daily in the
434 effluent. Consequently, the population inside the reactor is very active. Due to the
435 increase in biogas and methane generation when the HRT decreased, the activity
436 increased when the HRT decreased. In the single phase anaerobic system,
437 independently of the operated HRT, the positive correlation between activity and
438 methane generation was high. There was a high correlation between OLR and
439 microbial activity in single-stage anaerobic co-digestion of sewage sludge and sugar
440 beet pulp lixiviation.
441 In the TPAcD processes, the individual microbial activity of each phase decreased in
442 concordance with the reduction in methane generation at each stage. These results
443 seem to show that the activity of anaerobic microorganisms in the reactor could be
444 more related to the OLR than to microbial concentrations.
445 Under some conditions, microbial number and activity showed proportional
446 correlations, whereas this is not the case in many realistic circumstances. This requires

447 caution and critical thinking when one parameter is calculated or estimated from
448 another.
449 This study shows that the increase in microbial activity inside the reactor is directly
450 proportional to the OLR (or inversely proportional to the HRT) and inversely
451 proportional to the size of the microbial population in the system in single-stage
452 anaerobic co-digestion. These results are in accordance with those previously reported
453 by Solera et al. (2001b) [19], in contrast to results from other studies showing a direct
454 correlation between the methanogenic population and the organic loading rate [17,
455 34].
456 **4. Discussion**
457 Most full-scale biomethanation systems in use are single-stage mesophilic digesters, in
458 which it is difficult to provide optimal conditions for all four of the guilds of microbes.
459 As such, the metabolic activities of the microbial guilds are compromised and the
460 performance of single-stage mesophilic digesters is often suboptimal; the reduction of
461 TVS is rather slow and only a portion of TVS can be converted. Although pretreatments
462 using heat and diluted acid or base can improve the digestibility of the feedstock, they
463 inevitably increase capital and operational costs and potentially produce inhibitory
464 compounds. In addition, up to two thirds of the methane is produced from acetate in
465 anaerobic digesters [27], but syntrophic acetogens and acetoclastic methanogens have
466 extremely slow growth due to their thermodynamically unfavorable pathways [28].
467 Consequently, the entire biomethanation process in single-stage mesophilic AD
468 systems is often suboptimal and prone to being disrupted by the accumulation of
469 propionate and butyrate, especially at high organic loading rates [29].

Thermophilic AD is considered one of the most promising approaches to improve biomethanation by accelerating the hydrolysis of the polymeric feedstock and other metabolic pathways [30]. For microbial biomass-laden feedstocks, high temperatures help to lyse intact microbial cells, making the cellular components available for bioconversion. However, several studies have shown that thermophilic digesters suffer from poor stability due to the accumulation of VFA, especially propionate, reduced methane production, and an increased carbon dioxide content [29]. The above limitations associated with thermophilic AD are thought to be attributable to several factors. First, elevated temperatures decrease the diversity and robustness of methanogens in digesters, as only three species of methanogens have been identified in thermophilic anaerobic digesters [31]. Second, high temperature decreases the solubility of H_2. Third, some microbes, especially syntrophic acetogens and methanogens, are more susceptible to inhibitory metabolites (e.g., NH_3, H_2S, and propionic and butyric acids) at thermophilic temperatures than at mesophilic temperatures [27].

5. Conclusions

The single-stage mesophilic AcD was superior to the thermophilic AcD in terms of the specific methane yield, effluent quality and process stability. However, TVS reduction in the thermophilic AcD was higher than in the mesophilic AcD.

The performance of TPAcD was dependent on the HRT of the thermophilic digester, but the advantages of single-stage mesophilic and the thermophilic AD could be obtained in the TPAcD system. The effluent quality (in terms of specific methane yield and process stability) was higher for the TPAD process than for the single-stage mesophilic AcD, but not in terms of soluble COD and VFA. The TVS reduction in the

494 TPAcD process was much higher than in the single-stage mesophilic AcD and similar to
495 that in the single-stage thermophilic AcD.
496

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